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How omics technologies can contribute to the '3R' principles by introducing new strategies in animal testing

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In Europe, in light of ethical, political and commercial pressure, every effort should be made to replace animals with alternatives (e.g. *in vitro* models), to reduce the number of animals used in experiments to a minimum and to refine current testing strategies in a way that ensures animals undergo minimum pain and distress. Methods currently used in toxicology for mandatory safety tests rely heavily on the dosing of animals, followed by the detection and pathological evaluation of manifested toxic lesions. Through the integration of so-called 'omics' technologies, a global analysis of treatment-related changes on the molecular level becomes feasible and therefore might provide a means for predicting toxicity before classical toxicological endpoints. This Opinion article summarizes the key features of pushing the '3R' principles in animal testing, discusses the possible impact on safety testing in toxicology and describes the potential of using omics technologies for improved toxicity prediction to meet ethical, political and commercial expectations.

Animal testing for regulatory purposes: do we have alternatives?

The use of live animals for experiments has an important role in many forms of research; however, this gives rise to an ethical dilemma. On the one hand, most of the animals used are conscious and might be harmed by the experiments, whereas on the other hand, this research might potentially lead to a better understanding of human diseases. Although many people accept that the cause and treatment of serious diseases (e.g. cancer and cardiac infarction) should be investigated in animals, at the same time they do not accept animal experiments for safety testing of new cosmetics and chemicals (http://europa.eu.int/comm/health). At first glance, it is easy to agree on this position; however, problems become evident when examining this in more detail. If all new cosmetics and chemicals come into contact with our living environment without testing for their toxicity, this would dramatically increase the risks and hazards to human health and to the environment.

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Past experience has shown that it is possible to predict toxicity in human beings from animal experiments. For this reason, many animal tests were developed and acknowledged for human safety in the 1930s and 1940s. Because the toxicity testing accepted by the authorities in one country is rarely identical to those accepted in others, in 1982 the Organization for Economic Cooperation and Development (OECD) was the first international organization to agree on harmonized guidelines for the testing of chemicals (http://www.oecd.org). In 1990, the International Conference on Harmonisation (ICH) decided on a similar approach for the safety and efficacy testing of drugs (http://www.ich.org). The current animal experiments that are described in the guidelines and requested by regulatory agencies before chemicals or drugs come to the market are summarized in Table 1. The list of animal experiments is long because of the many different toxicity endpoints that have to be investigated for regulatory purposes (compare http://www.fda.gov with http://www.emea.eu.int).

At the same time, there has been growing pressure from the public to replace animal experiments with alternatives. The replacement of animals by *in vitro* techniques in safety testing would be preferable; however, the generally accepted alternative *in vitro* techniques are available for only a limited number of toxicity endpoints, such as mutagenicity, phototoxicity and skin corrosion [1]. Currently, several more *in vitro* tests that mimic toxicity endpoints such as acute toxicity, skin and/or eye irritation and photogenotoxicity are undergoing validation with the hope that these alternative tests can predict specific effects in human beings. Proving this is time and cost intensive and, thus, the biggest challenge for alternative methods in general. In addition, it has previously been a particular problem to go through the validation process and subsequently gain acceptance from the different regulatory agencies. For this reason, and to promote the implementation of alternatives to safety testing in animals, several EU member states have established centres for validation procedures: ZEBET (German National Centre for the Documentation and Evaluation of alternatives to Animal Experiments; http://www.bfr.bund.de); ECVAM (European Centre for the Validation of Alternative Methods; http://ecvam.jrc.it); and ICCVAM (Interagency Coordinating

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Table 1. Safety testing in animal experiments required by regulatory agencies and alternative *in vitro* tests

Test System	Alternative tests under evaluation	Validated test systems
Acute systemic toxicity	8	0
Eye irritation and corrosion	14	0
Skin irritation and corrosion	3	3
Phototoxicity	1	1
Metabolism and toxicokinetics	Several	0
Sub-acute and	Several	0
sub-chronic toxicity	Several	0
Skin sensitisation	3	3
Genotoxicity	12	0
Carcinogenicity	8	0

Committee for the Validation of Alternative Methods, http://iccvam.niehs.nih.gov). This reflects the global efforts to circumvent animal experiments whenever possible. Unfortunately, *in vitro* models have some drawbacks, which make their use as alternatives for animal experiments in safety testing questionable, at least in the near future. Generally, *in vitro* cultures represent complex organs by using only one or a limited number of cell types; therefore, they do not reflect organ integrity and thus cannot show the same treatment response to chemicals and drugs compared with the *in vivo* model. Furthermore, routine toxicity testing *in vivo* requires the histopathological investigation of >30 organs derived from one experimental animal. Consequently, to remodel the same situation using *in vitro* techniques, at least 30 different cell cultures would be necessary to represent the *in vivo* situation: this would make the alternatives expensive and time intensive. However, simplified *in vitro* systems can still be highly predictive if the key elements of the response have been unequivocally identified. Last, but not least, animal usage is highest when testing the prolonged exposure of chemicals and/or drugs for the prediction of long-term consequences, such as chronic toxicity, reproductive toxicity and cancer. These animal experiments are hard to mimic *in vitro* and might never be replaced but there is a chance to have them reduced or refined. Figure 1 illustrates the number of animals used in safety tests for different toxicity endpoints.

Why do we need to change safety testing in animals?

In 1959, Russell and Burch published their book *Principles of Humane Experimental Technique*, in which they described the '3Rs concept' (replacement, reduction and refinement) for the humane treatment of experimental animals [2]. As with many new ideas, the initial publication attracted little attention and was neglected by the scientific community for ~20 years. In the 1980s, these principles were established as essential considerations when animals are used in research and have finally influenced new legislation to control the use of experimental animals. In parallel, the development of alternatives to regulatory safety testing in animals has become the generally accepted scientific concept of government institutions such as ZEBET, ECVAM and ICCVAM. Nowadays, new legislation in Europe forces regulatory toxicity testing to apply the latest discoveries in molecular and cellular sciences to replace, reduce and/or refine animal experiments. The first push in this direction was the EU Cosmetics Directive, which phases out the use of animals in cosmetics safety testing over ten years (http://pharmacos.eudra.org). A short while later, the European Commission proposed its REACH legislation, a new directive for the registration, evaluation and authorisation of chemicals that will have a huge impact on, and might lead to a major change in, safety testing strategies for chemicals (http://europa.eu.int/comm/enterprise/reach). It is expected that the REACH regulation will be approved in 2006 and become effective in 2007. In Europe, ~30 000 chemicals are produced for the market, at more than one tonne per year, for which there has been no systematic submission of data to authorities. According to REACH, complete registration will be mandatory for both new and existing chemicals, even those that have been on the market for decades. To meet these regulatory requirements, significant toxicity data has to be generated for already existing

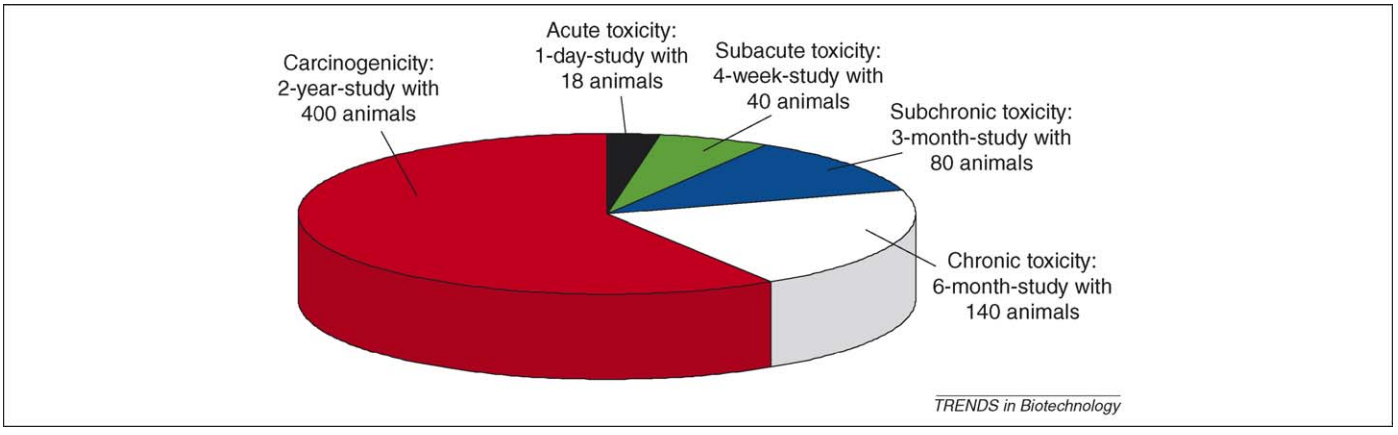

Figure 1. Average number of experimental animals used in mandatory safety tests in toxicology. Tests were selected according to different toxicity endpoints. Acute and sub-acute studies comprise between 60 and 70% of all studies.

chemicals, mainly by animal experiments. On the one hand, from an ethical, financial and practical point of view, the testing requirements for these chemicals are one of the most challenging for the chemical industry, requiring investigation of existing chemicals in millions of additional experimental animals. On the other hand, REACH offers an opportunity to innovate future safety testing strategies for chemicals and drugs. As mentioned before, the methods currently used to study the toxic effects of new chemicals and/or drugs rely mainly on classical safety studies in animals: histopathological examination of fixed and stained organ sections derived from animal experiments serves as one of the 'gold standards' for the detection of organ toxicity. For this purpose, experienced pathologists investigate the integrity of organs, cells and cellular subpopulations. Nevertheless, toxic lesions have to manifest in organs to enable detection, and the alterations at the molecular level preceding these morphological findings remain concealed. Therefore, histopathological evaluation might be advanced by new technologies that are able to detect molecular changes before the morphological changes occur. This has special relevance for long-term toxicity experiments and would open a window for shortening these studies in the future, thus reducing the number of animals and refining current experiments in safety testing. In addition, the limited possibility of extrapolating animal data to the human situation can be improved by using the innovative technologies [3].

The potential impact of omics technologies on safety testing

As illustrated in Figure 1, animal usage is highest in toxicity experiments representing prolonged exposure to chemicals or drugs for the prediction of long-term consequences, such as chronic toxicity, reproductive toxicity and cancer. At the same time, these animal experiments are hard to mimic *in vitro* and, for this reason, a replacement for the *in vivo* investigations is unlikely to be available in the near future. Furthermore, animal tests are often used as the 'gold standard' by which the *in vitro* test is measured. Even when the *in vitro* test is clearly better than the animal test, it takes a long time to implement owing to the problem of more rigorous evaluation, which the *in vivo* tests never have to face. However, there is an urgent demand for alternative methods to detect these toxicity endpoints earlier using fewer resources. Because the guidelines for assessing substances have been changed within the framework of the new chemical legislation in Europe (REACH concept), this demand is even more urgent than before. Data have to be produced for a large number of insufficiently characterised chemicals, and these data have to be collected from new animal studies unless alternative methods are available. Consequently, it is of great interest for risk and hazard assessment of human health to develop test systems permitting an improved prediction of the long-term toxic and carcinogenic potential of chemicals in short- to medium-term experiments [4,5].

Increasingly, new molecular methods (so-called omics technologies) are becoming available that hold the promise to detect tissue-specific changes with increasing sensitivity. These methods permit the simultaneous analysis of thousands of genes, proteins or metabolites and, thus, the global detection of treatment-related changes on the transcriptional and translational expression levels in animal experiments becomes possible (e.g. toxicogenomics, toxicoproteomics and metabolomics) [6–8]. By using these technologies, the chance arises to gain valuable information on the underlying toxicity mechanisms and potentially to fill the 'black hole' that exists between treatment and the classical morphological or clinical outcome [9]. Whether these methods are also able to detect molecular changes that are predictive of toxicity endpoints has to be proven. However, several publications have described the use of genomics and proteomics technologies to identify treatment-related alterations in gene or protein expression in tissues derived from short-term animal experiments. Ellinger-Ziegelbauer *et al.* showed that the characteristic gene expression profiles of genotoxic carcinogens, which are known to produce tumours in carcinogenicity studies, are already detectable in short-term *in vivo* studies. Fella *et al.* demonstrated the identification of potential protein biomarkers, which are predictive for liver cancer, after interim treatment of animals with a known carcinogen [10,11]. Furthermore, biofluids from experimental animals, evaluated by metabolomics technologies, showed significant changes in physiological metabolites after treatment with test compounds and were used to build up a database for the prediction of target-organ toxicity, mainly in the liver and kidney [12].

These examples illustrate the potential of omics technologies to predict specific endpoints of toxicity after short-term *in vivo* exposure in animals owing to the fact that these methods can detect even the smallest changes at the molecular level, preceding traditional morphological and clinical endpoints. In addition, the information on the pathways leading to toxic effects – the molecular mechanism – can be obtained from gene and/or protein expression profiles and metabolite patterns. This will be of great value in the assessment of the relevance of alterations seen in animals compared with humans. Moreover, one of the most important advantages of this strategy is the decreased duration of the safety testing, hence meeting the criteria for refinement of animal experiments. Consequently, it is possible that a 2-year study investigating carcinogenicity can, in the future, be shortened to a few weeks, resulting in an animal experiment with less pain and distress. Another advantage is the expected reduction in the number of animals required for such experiments. Although 50 rodents per dose group and sex would be treated and investigated for traditional carcinogenicity studies, only a few animals per dose group and sex might be sufficient for genomics, proteomics and metabolomic analyses for cancer prediction from short-term studies. This would lead to an enormous reduction in the number of animals used for testing carcinogenicity *in vivo*. The use of omics technologies in chronic toxicity tests might stimulate a similar challenge in refinement and reduction. Moreover, merging omics technologies with *in vivo* imaging tools might even lead to an increased gain of information. However, it should be mentioned that, so far, promising results have been obtained with only a small numbers of compounds. Omics technologies applied to toxicology suffer from sensitivity and

Box 1. Current limitations of omics implementation into classical toxicology

- Standardisation and annotation of research platforms and methods (e.g. MIAME)
- Pre-processing of raw data, choice and quality of algorithms
- Interlaboratory comparability of processed data
- Statistical analyses of processed data
- Interpretation of data (lack of standardized reference dataset)
- Relevance of results for tested organism
- Correlation of gene and/or protein expression changes with the potential for adverse effects
- Correlation of gene and/or protein expression changes with conventional parameters in toxicology

specificity problems; therefore, a broader spectrum of compounds need to be tested. Moreover, new safety-test strategies that integrate these technologies have to be funded, optimised, standardised, harmonised and validated before they will be accepted by regulatory authorities as alternatives. In spite of the fact that several promising innovative technologies have been available for years, they have not been sufficiently evaluated and validated with regard to their use in risk and/or hazard assessment, which shows that there is a serious scientific challenge to cope with, and, consequently, no prospective animal studies have been described. The current limitations of omics technologies are summarized in Box 1. Therefore, it is presently unjustifiable to substitute established regulatory safety studies in animals, such as chronic toxicity tests lasting 90 days or the 2-year carcinogenicity study, with short-term animal experiments analysed by omics technologies. Nevertheless, with the new EU legislation the unique opportunity should be taken for in-depth evaluation of omics technologies in short-term studies as a replacement for the more time- and animal-consuming safety testing experiments [13].

Concluding remarks

The Cosmetic Directive and the REACH legislation are expected to stimulate work towards the replacement of animals, refinement of animal experiments and reduction of the number of experimental animals needed to demonstrate the safety of chemicals. A few examples have been published, indicating that, by using omics technologies, risk and hazard assessments for chronic endpoints are possible from short-term animal experiments [10–12]. In addition, such a testing strategy might result in an increased gain of information from animal experiments, such as elucidating the underlying mechanisms, which would lead to an improved risk assessment. However, to prove any additional benefit of these technologies in safety testing, many substances will have to be tested in conventional animal experiments and, in parallel, analysed by innovative technologies. Sustained validation procedures have to follow before regulatory authorities will accept these technologies as alternatives. Nevertheless, the recent release of a draft guideline on the submission of pharmacogenomics data to the Food and Drug Administration (http://www.fda.gov/cder/guidance/5900dft.pdf), in combination with several other publications discussing the regulatory view of gene expression data [14–16], demonstrates that the potential of omics technologies has been considered by regulatory agencies and will influence their decision making in the future. For this reason, omics technologies should have the potential to lead a paradigm shift in safety testing. However, many challenges remain, and much work has to be done to bring the evidence that these alternative approaches are predictive for human toxicity.

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